THE EMBO JOURNAL

Manuscript EMBO-2012-82560

Structural basis for Arl3-specific release of myristoylated ciliary cargo from UNC119

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Review timeline:

Submission date:	04 July 2012
Editorial Decision:	01 August 2012
Revision received:	15 August 2012
Editorial Decision:	17 August 2012
Revision received:	20 August 2012
Accepted:	20 August 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 August 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, the referees are all positive about the paper and will support its publication here after appropriate revision. I would thus like to invite you to submit a revised version of the manuscript that addresses the points raised by the referees in an adequate manner.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The manuscript by Miertzschke et al. investigates the mechanisms of Arl3-mediated release of myristoylated ciliary cargo from UNC119. The authors demonstrate that although both Arl2 and Arl3 bind UNC119 with high affinity, only Arl3 is able to efficiently displace cargo from UNC119. Crystallographic analyses of Arl3-UNC119a and Arl2-UNC119a reveal the structure of these complexes and suggest that Arl3 utilizes an allosteric mechanism to weaken the interaction between UNC119a and its cargo by widening the cargo-binding pocket. The structures offer an explanation for the observation that cargo is releases by Arl3 but not Arl2 as the different modes of interactions in the Arl3-UNC119a and Arl2-UNC119a complexes affect the cargo-binding pocket in UNC119a differently. This model is corroborated by cargo-release of various mutant proteins. Finally, the authors explore the importance of the N-terminal helix of Arl3 in cargo release and show that it is indeed important in the release of tightly bound cargos. In agreement with this, a chimeric protein where the N-terminal helix of Arl3 is replaced with that of Arl2 is unable to release cargo. Overall, this is a very nice piece of work. The topic is highly novel as very little is known about the transport of proteins into the cilium. The mechanistic insights into cargo release from UNC119 by Arl3 are significant and well supported by the experiments presented. This study should be of high impact on the cilium field in general.

Comments/Issues:
The manuscript would benefit from editing and proofreading to improve clarity and readability. Several sentences are quite convoluted and hard to understand. Some suggestions are found below but there are many more typos and English language mistakes that I don't have the time to go over.

1) The two crystal structures of UNC119a in complex with either Arl2 or Arl3 were obtained from limited proteolysis (nothing wrong with that per se) but the identity of the proteolysed proteins were only analysed by N-terminal sequencing. As a number of regions in the complexes had no electron density visible (N-terminal 59 residues and more importantly the L9 region of UNC119a), this raises the question of whether these regions are disordered or have been proteolysed. In particular as the missing electron density for L9 in UNC119a is used to explain mechanistically the difference between the action of Arl2 and Arl3 it is important to assess if L9 is still intact in the proteolyzed Arl2-UNC119 complex or if the loop-region has been chopped. I suggest mass-spectrometry experiments on the proteolyzed complexes to address this issue.

Abstract, line 8: Contrary to previous structures from of GTP-bound...

Abstract, last sentence: This leads us to propose that ciliary targeting is not only dependent on nucleotide status but also on the spatial organization of Arl3.

This sentence seems to be too generalizing. There are many proteins targeted to the cilium that do not rely on Arl3 at all. Maybe insert 'ciliary targeting of 'lipidated (or 'prenylated/myristoylated) proteins'. By 'spatial organization' do you mean cellular localization?

Page 3, line 5: For the term ciliopathies maybe also cite an earlier review such as: Badano, J.L., Mitsuma, N., Beales, P.L. & Katsanis, N. (2006). The Ciliopathies: An Emerging Class of Human Genetic Disorders. Annu. Rev. Genom. Human Genet. 7, 125-148

Page 3, line 13: For PDEδ, UNC119a and UNC119b it has been shown that they contain a hydrophobic pocket which can that accommodates lipid moieties of post-translationally modified membrane-associated proteins

Page 4, first sentence of last paragraph: Here we show that Arl3 and but not Arl2...

Page 5, last sentence: as shown earlier for cystin by a solid phase binding assay.... reference missing

Page 5, line 20:...UNC119b and but not UNC119a...

Page 6, line 3: Arl3*GDP did not induce this effect (data not shown)... This seems to be quite an important point, I recommend that the authors show the data or refer to a proper reference if previously published.

Page 6, lines 4-7: this sentence contains several mistakes: The inability of Arl2*GppNHp to displace (a word seems to be missing, maybe peptide, cargo or GNAT1/NPHP3???) was not due to a weaker binding, as we have reported before and confirmed here (not shown) (if you don't show it here maybe just stick to as reported before) that the affinity of UNC119a for Arl2 is in fact very similar to those that if Arl3...

page 6, line 8 and figure 1b and d: In the case of UNC119b, Arl2*GppNHp seems to have some effect as the relative polarization changes. Maybe the authors can comment on this on pages 6/7.

Page 7, line 13: Crystallization of (full length?) Arl2 or Arl3 in complex with UNC119a was not successful.

Page 8, line 3: We obtained good well diffracting crystals...

Page 8, line 17: typical main-chain interactions.

Page 10, lines 11-13: The structural finding supports the results from proteolytic digestion where the N-terminal helix from Arl3•GppNHp but not Arl2•GppNHp was partially protected from proteolysis by complex formation with UNC119a.

This is a bit of an odd statement. I think it would make more sense to state that the crystal structure of the Arl3*GppNHp complex demonstrates that the N-terminal helix remains bound to the protein, which is supported by (or in agreement with) the proteolytic data.

Page 11, line 15: ...was reported to be critical for binding lipidated cargo (reference missing)

Page 11, last line: The reference is typed in 'symbol' font.

Page 12, line 12: ...flexibility of L9 is reflected by its poor electron density...
How do you know that it is flexible and not digested by your protease treatment?

Page 15, line 5: less strongly weaker

Page 15, line 8: ...is able to release Unc119-bound cargo only...

Page 18, line 15: How much glycerol was used as a cryo-protectant?

Page 18, line 16: replace Suisse with Swiss. Reference missing for XDS as well as for all other crystallographic programs used.

Page 18, line 20: One 'space' and '.' missing.

Table 1: The unit for B-factors is Å^2. The RMSD for bond length and angles are high (0.021-0.024Å and 2.0-2.1 degrees, respectively) for resolutions of 2.1-2.6Å. I suggest to change the X-ray versus geometry term in Refmac towards tighter geometry restraints for the coordinates that will be submitted to the PDB.

Referee #2

The present manuscript by Ismail et al. describes a continuation of previous studies by the same group addressing the molecular mechanism of Arl-mediated protein trafficking and membrane targeting. Arl2 and Arl3 are closely related small G proteins that utilize an allosteric mechanism to release lipidated cargo from GDI-like solubilizing factors. Here, the authors focus on Arl2 and Arl3's interaction with UNC119, a protein responsible for the delivery of myristoylated cargo to ciliary membranes. While both G proteins bind to UNC119, only Arl3 is capable of triggering significant cargo release from UNC119. A comparison of complex crystal structures (UNC119•Arl2•GppNHp and UNC199•Arl3•GppNHp) provides a molecular explanation for the apparent specificity, and also reveals differences in mechanism compared to PDEdelta, another GDI-like solubilizing factor that is regulated by the two G proteins.

In summary, the manuscript describes an interesting mechanism for the specificity encoded in Arl3-mediated cargo release, which suggests a model for ciliary-specific targeting of proteins. The work is based on two novel crystal structures and a thorough biophysical validation.

My main issue is of stylistic nature. I would have preferred having the discussion of the interfaces as part of the initial, basic characterization (after Figure 2), followed by the analysis of the molecular mechansim. In the present manuscript, the authors inserted the molecular mechanism (opening of the myristoyl binding pocket) as a middle section, breaking the interface description into two parts. In addition, more extensive labeling of the structural figures may increase the readability by relying less on the figure legends. For example, label the interswitch toggle in Figure 1; the loop 9 in Figure 4A.

Additional points:
1. Figures 1 and 5: In the release assays, only a fraction of the cargo (GNAT1) is released from UNC119a upon Arl3 addition, and there is no apparent off-rate for the remainder. This may suggest the presence of two different populations of cargo-UNC119a complexes. In contrast, weakening cargo interactions by mutation enables almost quantitative release. What could be a molecular explanation for the distinct results?
2. Since the authors use one-letter codes for the protein residue labels, designation "L9" for loop 9 is confusing.
3. Out of curiosity, I was wondering if the authors attempted to make an N3-Arl2 fusion? In the manuscript, only an N2-Arl3 fusion was described, which is a loss of function mutation, whereas one may expect a gain of function from N3-Arl2.
4. What is the explanation for the difference in plateau height (point of saturation) in the binding experiment shown in Supplemental Figure 2C?
5. The mechanism and analysis of pocket opening in UNC119a was carried out with the Unc119a/GNAT-1 structure as a reference. The Arl2-containing complex was not included since loop 9 is disordered in this structure. Since Arl2 does not release cargo, one would expect that the myristoyl binding pocket is still closed, and that the apparent disorder of loop 9 does not affect cargo binding/release. Yet, a lot of the discussion pertains to the structure of loop 9 in the complex containing Arl3. Hence, an analysis of the Arl2-containing complex may strengthen (or weaken) the validity of the proposed mechanism. Please comment.
6. Since the protease protection assays are a central part of the structural argument and support the notion that the amphipathic helix is partially buried in the Arl3-containing complex, I would suggest including the figure in the main manuscript (right now, the data are not shown).
7. The model for specific cargo release from UNC119 and how it relates to membrane binding and GAP interaction (last two paragraphs of the discussion) is not very clear. Maybe a cartoon would help to illustrate the model.
8. The running title is misleading since the authors do not investigate ciliary targeting directly.
9. Please check the text and figures for consistency (e.g. UNC119 or Unc119; capital vs. lower case letter in the figure legends).

Referee #3

This is an excellent manuscript that provides a highly detailed explanation for why Arl3-GTP but not Arl2-GTP dissociates cilia-destined cargoes from Unc119. There was a recent flurry of papers

showing that Unc119 recognizes specific myristoylated cargoes and that Arl3-GTP releases those cargoes (presumably in the cilium). However, it remained puzzling that this effect was restricted to Arl3 given its very close similarty to Arl2. Now Wittinghofer and colleagues make a very clear case that both Arl2 and Arl3 bind Unc119 when GTP bound but only Arl3 has the ability to release cargo. Surprisingly Arl3 does not eject its amphipathic N terminus upon GTP binding but instead reposition the so-called "interswitch toggle" (an internal beta hairpin) to give a gentle shove to unc119. This contact triggers an allosteric enlargement of the myristoyl-binding pocket that dramatically decrases the affinity of unc119 to its cargoes. The experimental quality is exceptional throughout and the authors present their results in an intelligible fashion to make the paper readable by the broadest audience. Many of the findings are novel and unexpected and will be of interest to GTPase researchers and to people interested in mechanisms of transport to cilia.
I recommend publication in the current form.

Minor points:
p.7: The first sentence claims that "Crystallizing [..] Arl3 in complex with Unc119a was not successful" then on p.8 go on to say that they "obtained good diffracting crystals for the complexes [of Unc119a] with Arl3•GppNHp". The first sentence may need to be properly qualified.

p.11: the reference after PDE∂ on the last line is in greek characters.

P.13: last paragraph. "diverse" should be "divergent"

1st Revision - authors' response 15 August 2012

Referee #1

The manuscript would benefit from editing and proofreading to improve clarity and readability. Several sentences are quite convoluted and hard to understand. Some suggestions are found below but there are many more typos and English language mistakes that I don't have the time to go over

We included all the suggestions and corrected the typos

The two crystal structures of UNC119a in complex with either Arl2 or Arl3 were obtained from limited proteolysis (nothing wrong with that per se) but the identity of the proteolysed proteins were only analysed by N-terminal sequencing. As a number of regions in the complexes had no electron density visible (N-terminal 59 residues and more importantly the L9 region of UNC119a), this raises the question of whether these regions are disordered or have been proteolysed. In particular as the missing electron density for L9 in UNC119a is used to explain mechanistically the difference between the action of Arl2 and Arl3 it is important to assess if L9 is still intact in the proteolyzed Arl2-UNC119 complex or if the loop-region has been chopped. I suggest mass-spectrometry experiments on the proteolyzed complexes to address this issue.

We now performed mass-spectrometry after gel electrophoresis on the tryptically digested Arl3GppNHpUNC119 and Arl2GppNHpUNC119. Peptides containing loop 9 were detected in the mass spectrometry in both complexes, however we could not detect peptides from the N-terminal 57 amino acids. This confirms our previous conclusion that the lower molecular weight unc119 is due to the digestion of the N-terminal amino acids and that the poor electron density of loop 9 in the Arl2GppNHpUNC119 structure is due to flexibility of the loop and not digestion. We mention this in the main text page 12 lines 16-17)

Page 6, line 3: Arl3*GDP did not induce this effect (data not shown)... This seems to be quite an important point, I recommend that the authors show the data or refer to a proper reference if previously published.

This data is now included in supplementary figure one as panel d

page 6, line 8 and figure 1b and d: In the case of UNC119b, Arl2*GppNHp seems to have some effect as the relative polarization changes. Maybe the authors can comment on this on pages 6/7

Unc119b seems to be in general more prone to cargo release. As Arl3 releases Unc119b cargo more efficient than in case of Unc119a, and even with Arl2 we can see a mild release, we highlighted this observation in Page 6 lines 12-16

The RMSD for bond length and angles are high (0.021-0.024Å and 2.0-2.1 degrees, respectively) for resolutions of 2.1-2.6Å. I suggest to change the X-ray versus geometry term in Refmac towards tighter geometry restraints for the coordinates that will be submitted to the PDB

We used tighter geometry restraints and now the RMSD for bond length and angles are 0.008-0.016 Å and 1.147-1.714° for resolutions of 2.1-2.6Å.

Referee #2

My main issue is of stylistic nature. I would have preferred having the discussion of the interfaces as part of the initial, basic characterization (after Figure 2), followed by the analysis of the molecular mechanism. In the present manuscript, the authors inserted the molecular mechanism (opening of the myristoyl binding pocket) as a middle section, breaking the interface description into two parts. In addition, more extensive labeling of the structural figures may increase the readability by relying less on the figure legends. For example, label the interswitch toggle in Figure 1; the loop 9 in Figure 4A.

We wanted to present the structural explanation of Arl3 specificity at the end of the manuscript, since the specificity is based on the structure of the interface and the opening of the pocket that we decided to describe first. We thus kept the order of descriptions.

We now more extensively label the structural figures, including the interswitch toggle in figure 1 and the loop 9 in figure4A

Figures 1 and 5: In the release assays, only a fraction of the cargo (GNAT1) is released from UNC119a upon Arl3 addition, and there is no apparent off-rate for the remainder. This may suggest the presence of two different populations of cargo-UNC119a complexes. In contrast, weakening cargo interactions by mutation enables almost quantitative release. What could be a molecular explanation for the distinct results?

This is indeed true. We show in the current study that Arl3 releases the UNC119 cargo allosterically and by forming a fast dissociating complex. Hence it is not a pure competition which means that Arl3 modulates the affinity of the cargo to UNC119 and therefore, as the referee rightfully mentions, at equilibrium we have a mixed population of binary, ternary complexes in addition to the released peptides. In case of the mutants that have lower affinities to cargo, more cargo will be will released at equilibrium under the conditions used. We now describe this situation in page 6 lines 4-5.

Since the authors use one-letter codes for the protein residue labels, designation "L9" for loop 9 is confusing
We now designated "L9" as "l9"

Out of curiosity, I was wondering if the authors attempted to make an N3-Arl2 fusion? In the manuscript, only an N2-Arl3 fusion was described, which is a loss of function mutation, whereas one may expect a gain of function from N3-Arl2.

We now did this experiment. However the N3-Arl2 fusion protein was not able to release the cargo indicating that although the amino acid sequence of Arl3 N terminal helix is required for cargo release, it is not sufficient for cargo release when present in Arl2. This suggests that other regions of the protein stabilize the Arl3 helix conformation. We mention and discuss the result of this experiment on page 14 lines 14-18

What is the explanation for the difference in plateau height (point of saturation) in the binding experiment shown in Supplemental Figure 2C?

There is indeed a difference in the fluorescence polarization values between DArl3mantGppNHpUNC119a and Arl3GppNHpUNC119. This difference could be due to different fluorophore environment especially that we are expecting a conformation change between the DArl3mantGppNHpUNC119a and Arl3GppNHpUNC119. However this difference in absolute polarization value would not have any effect on the titration and the affinity values.

The mechanism and analysis of pocket opening in UNC119a was carried out with the Unc119a/GNAT-1 structure as a reference. The Arl2-containing complex was not included since loop 9 is disordered in this structure. Since Arl2 does not release cargo, one would expect that the myristoyl binding pocket is still closed, and that the apparent disorder of loop 9 does not affect cargo binding/release. Yet, a lot of the discussion pertains to the structure of loop 9 in the complex containing Arl3. Hence, an analysis of the Arl2-containing complex may strengthen (or weaken) the validity of the proposed mechanism. Please comment.

Our explanation of the specificity is based on the finding that Arl3 stabilizes a widened pocket conformation that does not favor cargo binding. However in the case of the Arl2 complex several interface contacts are lost and this conformation is not stabilized. This makes loop 9 as part of the binding pocket flexible such that we don't see it in the structure. We do (and did) agree with the referee that the pocket should be closed in the ternary complex (page 12 , lines 16,17).

Since the protease protection assays are a central part of the structural argument and support the notion that the amphipathic helix is partially buried in the Arl3-containing complex, I would suggest including the figure in the main manuscript (right now, the data are not shown).

The difference in molecular weight between the digested and protected Arl3 and Arl2 is very small (10 amino acids) and we did not observe difference in migration on gel electrophoresis. The difference is only seen by N terminal sequencing thus the gel electrophoresis is non-informative.

The model for specific cargo release from UNC119 and how it relates to membrane binding and GAP interaction (last two paragraphs of the discussion) is not very clear. Maybe a cartoon would help to illustrate the model.

A cartoon diagram explaining the model is now added as figure 7.

The running title is misleading since the authors do not investigate ciliary targeting directly.

We now changed the title

We corrected other typos suggested by the referee

Referee #3

We corrected the typos and corrected the reference as pointed out

2nd Editorial Decision 17 August 2012

Thank you for sending us your revised manuscript. I have now had a chance to look at the revisions you made, and it is clear that you have addressed all criticisms in a satisfactory manner.

Prior to formal acceptance, I need to ask to send us an amended version of the manuscript text file that includes the PDB accession details. We will upload the amended file for you and formally accept the manuscript at that point.

Thank you very much.

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - authors' response 20 August 2012

Thank you very much for your e-mail. Please find attached an amended version of the manuscript text file which includes the PDB accession details.

